## Use of malate dehydrogenase for NADH regeneration

The present invention relates to a process for the
preparation of enantiomerically enriched organic compounds.
In particular, the present invention relates to an
5   enzymatically operating process, in which, in a coupled
enzymatic reaction system, NAD(P)H is consumed by one
enzyme for the preparation of the organic compound and the
NAD(P)H is simultaneously regenerated by a second enzyme
system.
10 A reaction system which operates according to the invention
in this manner and an advantageous whole cell catalyst or
suitable plasmids are also proposed.

The production of optically active organic compounds, e.g.
alcohols and amino acids, by a biocatalytic route is
15 increasingly gaining importance. The coupled use of two
dehydrogenases with cofactor regeneration has emerged,
inter alia, as a route for the large-scale industrial
synthesis of these compounds, in particular alcohols and
amino acids (DE19753350, EP118750).

20 Equation 1:
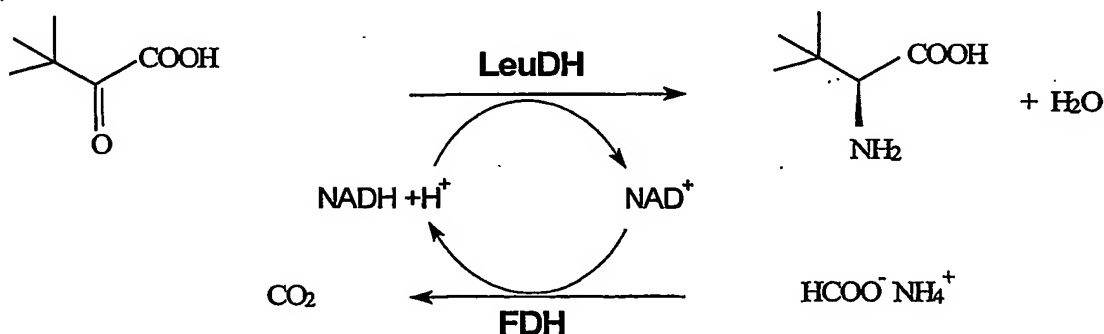
In situ regeneration of NADH with the NAD-dependent formate
dehydrogenase from Candida boidinii in the reductive
amination of trimethyl pyruvate to give L-tert-leucine
25 (Bommarius et al. Tetrahedron Asymmetry 1995, 6, 2851-
2888).

In addition to their catalytic property and efficiency, the biocatalysts efficiently employed in an aqueous medium furthermore have the advantage that in contrast to a large number of synthetic metal-containing catalysts, the use of

5   metal-containing starting substances, in particular those which contain heavy metals and are therefore toxic, can be dispensed with. The use of expensive and furthermore hazardous reducing agents, such as, for example, borane, in the case of asymmetric reduction can also be dispensed

10   with.

The FDH, e.g. from *Candida boidinii*, successfully employed to date in these systems has the disadvantage that the specific activity of this enzyme class at 4-8 U/mg is very low. This necessitates the use of a large amount of

15   expensive enzyme with recycling which is difficult from the apparatus point of view if a process designed in this way is to be carried out advantageously under economic aspects on an industrial scale.

Malate dehydrogenase (MDH), called "malic enzyme" catalyses

20   the oxidative decarboxylation of malate to pyruvate. Numerous malate dehydrogenases from various organisms are known, thus, inter alia, from higher animals, plants and microorganisms. A distinction is made between four types of malate dehydrogenases, which are classified into the enzyme

25   classes E.C. 1.1.1.37 to E.C. 1.1.1.40 (http://www.genome.ad.jp). NAD and/or NADP is required as a cofactor, depending on the type of malate dehydrogenase.

On the basis of the irreversibility of the oxidative decarboxylation reaction of L-malic acid to pyruvate, the

30   use of malate dehydrogenase is also appropriate in the systems described above in respect of a favourable cofactor regeneration.
The use of malate dehydrogenase for regeneration of NAD is described, for example, by Suye et al. in a work from 1992

35   (S.-I. Suye, M. Kawagoe, S. Inuta, *Can. J. Chem. Eng.* **1992**,

70, 306-312). NADH regeneration by means of malate
dehydrogenase is used here for reductive amination of
pyruvate by means of an alanine dehydrogenase, NADH being
consumed. The pyruvate is formed by oxidative

5   decarboxylation from L-malic acid, and is immediately
consumed again in the following step by alanine
dehydrogenase. A concentration of pyruvate in the reaction
solution is therefore avoided, whereby the problem of any
inhibitions of the enzymes involved by the presence of

10 amounts of pyruvate in the stoichiometric range is also
eliminated. Nevertheless, the system described is limited
exclusively to the production of alanine (see also S.-I.
Suye, *Recent Res. Devel. Ferment. Bioeng.* **1998**, *1*, 55-64).

Interestingly - in spite of the technically high potential

15 - apart from these works by the Suye study group no further
works using "malate dehydrogenase" for regeneration of the
oxidized cofactor NAD+ during reductions of ketones or
reductive aminations of keto acids have been described.

The object of the present invention was therefore to

20 provide a further process for the preparation of chiral
organic compounds, such as amino acids or alcohols, which
can be obtained by a coupled enzymatic reaction system as
described above, using malate dehydrogenase, which is not
limited to the preparation of one substance. In particular,

25 it should be possible to employ this process on an
industrial scale particularly advantageously under economic
and ecological aspects.

This object is achieved by a process with the
characterizing features of claim 1 of the present

30 invention. Claims 2 to 7 relate to preferred embodiments.
Claims 8 and 9 protect a reaction system according to the
invention and a correspondingly operating whole cell
catalyst. Claim 10 protects preferred plasmids.

In a process for the preparation of enantiomerically
enriched organic compounds in a coupled enzymatic reaction
system comprising a first enzymatic transformation of an
organic substrate, NAD(P)H being consumed, and the
5   regeneration of the NAD(P)H in a second enzymatic
transformation by a malate dehydrogenase, with oxidation of
L-malic acid to pyruvate and CO_2, as a result of not
employing the pyruvate formed from the second enzymatic
transformation as the substrate in the first enzymatic
10   transformation, the object described is achieved in an
entirely surprising but in return no less advantageous
manner. It can be regarded entirely as surprising that the
simultaneous use e.g. of an amino acid dehydrogenase with
malate dehydrogenase, which has a decarboxylating action,
15   is possible without problems without the occurrence of
cross-reactions. In addition, no inhibiting influences of
substrates and products of the primary reaction on the
malate dehydrogenase and vice versa can be detected. In
particular, it is a positive feature that the pyruvate
20   formed as the by-product does not have an inhibiting action
on the malate dehydrogenase itself or on the alcohol or
amino acid dehydrogenases employed in parallel.

Enantiomerically enriched alcohols or amino acids are
advantageously prepared with the process according to the
25   invention. In this case, inexpensive alcohol dehydrogenase
or amino acid dehydrogenase which are known universally are
possible as enzymes for the first enzymatic transformation.
The expert is in principle free in the choice thereof,
which is made according to the nature of the substrate
30   spectrum, stability and rate of conversion of the enzyme in
question. Known enzymes of this origin are described in K.
Drauz, H. Waldmann (eds.), Enzyme Catalysis in Organic
Synthesis, volume III, Wiley-VCH, Weinheim, **2002**, chapter
15.
35   The use of the alcohol dehydrogenase from the organisms
Rhodococcus erythropolis (S-ADH) or *Lactobacillus kefir* (R-

ADH) (ADH from *R. erythropolis*: J. Peters, T. Zelinski, M.-
R. Kula, Purification and characterization of a novel
carbonyl reductase silated from *Rhodococcus erythropolis*,
J. Biotechnol. 1994, 33, 283-292)(ADH from *Lactabacillus*
5   *kefir*: C. W. Bradshaw, W. Hummel, C.-H. Wong, *Lactobacillus
kefir* Alcohol Dehydrogenase: A Useful Catalyst for
Synthesis, J. Org. Chem. 1992, 57, 1532-1536.) is
particularly advantageous. In respect of preferred amino
acid dehydrogenases, the expert is referred to, for
10   example, leucine dehydrogenases or phenylalanine
dehydrogenases (A. Bommarius in: Enzyme Catalysis in
Organic Synthesis (eds.: K. Drauz, H. Waldmann), volume
III, Wiley-VCH, Weinheim, **2002**, chapter 15.3).

Malate dehydrogenases are also familiar to the expert (lit.
15   see above or the dissertation by S. Naamnieh, University of
Düsseldorf, in preparation). Here also, the expert will
choose the dehydrogenase which can be employed most
efficiently for his purpose. In principle, those malate
dehydrogenases which regenerate the NAD(P)H in an extent
20   such that no bottleneck arises for the course of the
reaction of the other enzyme employed.
The known malate dehydrogenase from *E. coli* K12 is
preferred in this connection. Gene isolation and cloning
are described in S. Naamnieh's dissertation, University of
25   Düsseldorf, in preparation p. 70 et seq.

In principle, the process according to the invention can be
carried out in purely aqueous solution. However, it is also
possible to add any desired parts of a water-soluble
organic solvent to the aqueous solution in order e.g. to
30   optimize the reaction in respect of poorly water-soluble
substrates. Possible such solvents are, in particular,
ethylene glycol, DME or glycerol.
However, multi-phase, in particular two-phase systems
comprising an aqueous phase can furthermore also serve as
35   the solvent mixture for the process according to the

invention. The use of certain solvents which are not water-
soluble has already proved itself here (DE10233107). The
statements made there in this respect also apply here
accordingly.

5   The expert in principle has a free choice of the
temperature present during the reaction. He preferably
directs himself towards obtaining a highest possible yield
of product in the highest possible purity in the shortest
possible time. Furthermore, the enzymes employed should be
10   sufficiently stable under the temperatures employed and the
reaction should proceed with the highest possible
enantioselectivity. In respect of the use of enzymes from
thermophilic organisms, it is entirely possible for
temperatures of 100°C to represent the upper limit of the
15   temperature range in the reaction. -15°C is certainly
appropriate as the lower limit in aqueous systems. A
temperature interval between 10 and 60, particularly
preferably between 20 and 40°C is advantageously to be
established.

20   The pH during the reaction is determined by the expert from
the enzyme stabilities and conversion rates and is adjusted
accordingly for the process according to the invention. For
the malate dehydrogenase from *E. coli* it has been found
that the optimum pH is > 10. In general, the range
25   preferred for enzymes from pH 5 to 11 is chosen. A pH range
from 5.5 to 10.0, in particular 6.0 to 9.0, can preferably
be present.

The invention also provides a coupled enzymatic reaction
system for the preparation of enantiomerically enriched
30   organic compounds, comprising a first enzymatic
transformation of an organic substrate, NAD(P)H being
consumed, and the regeneration of the NAD(P)H in a second
enzymatic transformation by a malate dehydrogenase, with
oxidation of L-malic acid to pyruvate and CO_2, wherein the
35   pyruvate formed from the second enzymatic transformation is

not employed as the substrate in the first enzymatic
transformation. The same advantages and preferred
embodiments as have already been mentioned in respect of
the process according to the invention apply in principle
5   to this reaction system.
The reaction system is advantageously employed, for
example, in a stirred tank, a cascade of stirred tanks or
in membrane reactors, which can be operated both in batch
operation and continuously.
10   In the context of the invention, membrane reactor is
understood as meaning any reaction vessel in which the
catalyst is enclosed in a reactor, while low molecular
weight substances are fed to the reactor or can leave it.
The membrane here can be integrated directly into the
15   reaction space or incorporated outside in a separate
filtration module, in which the reaction solution flows
continuously or intermittently through the filtration
module and the retained product is recycled into the
reactor. Suitable embodiments are described, inter alia, in
20   WO98/22415 and in Wandrey et al. in Yearbook 1998,
Verfahrenstechnik und Chemieingenieurwesen, VDI p. 151 et
seq.; Wandrey et al. in Applied Homogeneous Catalysis with
Organometallic Compounds, vol. 2, VCH 1996, p. 832 et seq.;
Kragl et al., Angew. Chem. 1996, 6, 684 et seq.
25   The continuous procedure which is possible in this
apparatus, in addition to the batch and semi-continuous
procedure, can be carried out here as desired in the cross-
flow filtration mode (fig. 3) or as dead-end filtration
(fig. 2). Both process variants are described in principle
30   in the prior art (Engineering Processes for Bioseparations,
ed.: L.R. Weatherley, Heinemann, 1994, 135-165; Wandrey et
al., Tetrahedron Asymmetry 1999, 10, 923-928).

The present invention also provides whole cell catalysts
comprising a cloned gene for a first enzyme for
35   transformation of an organic substrate and a cloned gene
for a malate dehydrogenase, these being capable of

preparation of an enantiomerically enriched organic
compound in a first enzymatic transformation, NAD(P)H being
consumed, and of allowing the regeneration of the NAD(P)H
to take place in a second enzymatic transformation by
5   malate dehydrogenase, with oxidation of L-malic acid to
pyruvate and CO_2, wherein the pyruvate formed from the
second enzymatic transformation is not employed as the
substrate in the first enzymatic transformation.
The whole cell catalyst according to the invention
10   preferably has an enzyme (polypeptide) with amino acid or
alcohol dehydrogenase activity and one with malate
dehydrogenase activity, originating in particular from the
organisms mentioned above.
Microorganisms which can be used are in principle all the
15   organisms possible to the expert for this purpose, such as
e.g. yeasts, such as *Hansenula polymorpha*, *Pichia sp.* and
Saccharomyces cerevisiae, prokaryotes, such as *E. coli* and
Bacillus subtilis or eukaryotes, such as mammalian cells
and insect cells. *E. coli* strains are preferably to be used
20   for this purpose. The following are very particularly
preferred: *E. coli* XL1 Blue, NM 522, JM101, JM109, JM105,
RR1, DH5α, TOP 10⁻ or HB101.
An organism as mentioned in DE10155928 is preferably
employed as the host organism.
25   The advantage of such an organism is the simultaneous
expression of both polypeptide systems, which means that
only one rec-organism has to be cultured for the reaction
according to the invention.
To coordinate the expression of the polypeptides in respect
30   of their rates of conversion, the corresponding coding
nucleic acid sequences can be accommodated on different
plasmids with different numbers of copies and/or promoters
of different potency can be used for an expression of the
nucleic acid sequences of different intensity. In such
35   coordinated enzyme systems, advantageously no accumulation
of an intermediate compound occurs, and the reaction in
question can proceed at an optimum overall rate. However,

this is adequately known to the expert (Gellissen, G.;
Piontek, M.; Dahlems, U.; Jenzelewski, V.; Gavagan, J. W.;
DiCosimo, R.; Anton, D. L.; Janowicz, Z. A. (1996),
Recombinant Hansenula polymorpha as a biocatalyst.
5   Coexpression of the spinach glycollate oxidase (GO) and the
S. cerevisiae catalase T (CTT1) gene, Appl. Microbiol.
Biotechnol. 46, 46-54; Farwick, M.; London, M.; Dohmen, J.;
Dahlems, U.; Gellissen, G.; Strasser, A. W.; DE19920712).
It is very particularly advantageous that the whole cell
10 catalyst according to the invention optionally further
metabolizes the pyruvate formed in the reaction according
to the invention, as it may uses it as a nutrient source.
Whole cell catalysts designed in this manner mean that the
pyruvate is not obtained as a by-product of the reaction
15 and therefore also does not have to be separated off from
the chiral product actually desired in further process
steps.
The preparation of the whole cell catalyst can in principle
be carried out by measures known to the expert (Sambrook,
20 J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular
cloning: a laboratory manual, 2nd ed., Cold Spring Harbor
Laboratory Press, New York; Balbas, P. and Bolivar, F.
(1990), Design and construction of expression plasmid
vectors in E. coli, Methods Enzymol. 185, 14-37; Rodriguez,
25 R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of
molecular cloning vectors and their uses, 205-225,
Butterworth, Stoneham). In respect of the general procedure
(PCR, cloning, expression etc.) reference may also be made
to the following literature and that cited there: Universal
30 GenomeWalker™ Kit User Manual, Clontech, 3/2000 and
literature cited there; Triglia T.; Peterson, M. G. and
Kemp, D.J. (1988), A procedure for in vitro amplification
of DNA segments that lie outside the boundaries of known
sequences, Nucleic Acids Res. 16, 8186; Sambrook, J.;
35 Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning:
a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory
Press, New York; Rodriguez, R.L. and Denhardt, D. T (eds)

(1988), Vectors: a survey of molecular cloning vectors and
their uses, Butterworth, Stoneham.

The invention also provides plasmids containing gene
constructs in which the gene for a malate dehydrogenase and
5   a gene for an enzyme for transformation of an organic
substrate with consumption of NAD(P)H are present.
Possible plasmids or vectors of origin are in principle all
the embodiments available to the expert for this purpose.
Such plasmids and vectors can be found e.g. in Studier and
10   colleagues (Studier, W. F.; Rosenberg A. H.; Dunn J. J.;
Dubendroff J. W.; (1990), Use of the T7 RNA polymerase to
direct expression of cloned genes, Methods Enzymol. 185,
61-89) or the brochures of Novagen, Promega, New England
Biolabs, Clontech or Gibco BRL. Further preferred plasmids
15   and vectors can be found in: Glover, D. M. (1985), DNA
cloning: A Practical Approach, vol. I-III, IRL Press Ltd.,
Oxford; Rodriguez, R.L. and Denhardt, D. T (eds) (1988),
Vectors: a survey of molecular cloning vectors and their
uses, 179-204, Butterworth, Stoneham; Goeddel, D. V.
20   (1990), Systems for heterologous gene expression, Methods
Enzymol. 185, 3-7; Sambrook, J.; Fritsch, E. F. and
Maniatis, T. (1989), Molecular cloning: a laboratory
manual, 2nd ed., Cold Spring Harbor Laboratory Press, New
York.
25   Plasmids with which the gene construct containing the
nucleic acids according to the invention can be cloned into
the host organism in a very preferred manner are: pUC18
(Roche Biochemicals), pKK-177-3H (Roche Biochemicals),
pBTac2 (Roche Biochemicals), pKK223-3 (Amersham Pharmacia
30   Biotech), pKK-233-3 (Stratagene) or pET (Novagen). The
plasmid pkk/phe/mali (fig. 5) is advantageous in this
connection.
The preparation of this plasmid and of a corresponding rec-
microorganism is described in the dissertation by S.
35   Naamnieh, University of Düsseldorf, in preparation p. 70 et
seq.

For the use, the polypeptides in question of the process
according to the invention can be used in the free form as
homogeneously purified compounds or as an enzyme prepared
by a recombinant method. These polypeptides can furthermore
5   also be employed as a constituent of an intact guest
organism or in combination with the broken-down cell mass
of the host organism, which has been purified to any
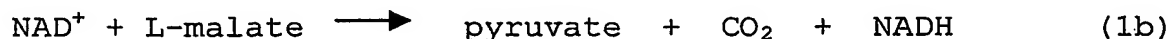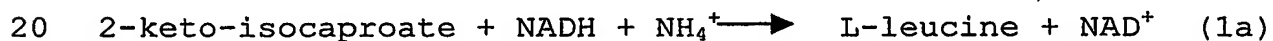desired extent.
The use of the enzymes in immobilized form is also possible
10   (Sharma B. P.; Bailey L. F. and Messing R. A. (1982),
Immobilisierte Biomaterialiern - Techniken und
Anwendungen, Angew. Chem. 94, 836-852). The immobilization
is advantageously carried out by lyophilization (Paradkar,
V. M.; Dordick, J. S. (1994), Aqueous-Like Activity of α-
15   Chymotrypsin Dissolved in Nearly Anhydrous Organic
Solvents, J. Am. Chem. Soc. 116, 5009-5010; Mori, T.;
Okahata, Y. (1997), A variety of lipi-coated glycoside
hydrolases as effective glycosyl transfer catalysts in
homogeneous organic solvents, Tetrahedron Lett. 38, 1971-
20   1974; Otamiri, M.; Adlercreutz, P.; Matthiasson, B. (1992),
Complex formation between chymotrypsin and ethyl cellulose
as a means to solubilize the enzyme in active form in
toluene, Biocatalysis 6, 291-305). Lyophilization in the
presence of surface-active substances, such as Aerosol OT
25   or polyvinylpyrrolidone or polyethylene glycol (PEG) or
Brij 52 (diethylene glycol monocetyl ether) (Kamiya, N.;
Okazaki, S.-Y.; Goto, M. (1997), Surfactant-horseradish
peroxidase complex catalytically active in anhydrous
benzene, Biotechnol. Tech. 11, 375-378), is very
30   particularly preferred.
Immobilization on Eupergit® , in particular Eupergit C® and
Eupergit 250L® (Röhm) (Eupergit.RTM. C, a carrier for
immobilization of enzymes of industrial potential.
Katchalski-Katzir, E.; Kraemer, D. M. Journal of Molecular
35   Catalysis B: Enzymatic (2000), 10(1-3), 157-176), is
extremely preferred.
Immobilization on Ni-NTA in combination with the

polypeptide supplemented with the His tag (hexa-histidine)
is likewise preferred (Purification of proteins using
polyhistidine affinity tags. Bornhorst, Joshua A.; Falke,
Joseph J. Methods in Enzymology (2000), 326, 245-254).

5 The use as CLECs is also conceivable (St. Clair, N.; Wang,
Y.-F.; Margolin, A. L. (2000), Cofactor-bound cross-linked
enzyme crystals (CLEC) of alcohol dehydrogenase, Angew.
Chem. Int. Ed. 39, 380-383).
By these measures it can be possible to generate from

10 polypeptides which become unstable due to organic solvents
those which can operate in mixtures of aqueous and organic
solvents or entirely in organic media.

The process according to the invention can be carried out
such that the MDH from *E. coli* is coupled with an NAD-

15 dependent leucine dehydrogenase (LeuDH from *Bacillus
cereus*; Sigma). LeuDH catalyses the reductive amination of
aliphatic keto acids, such as, for example,
ketoisocaproate, to the corresponding L-amino acids, such
as L-leucine, with consumption of NADH (equation (1)).

20 2-keto-isocaproate + NADH + $NH_4^+ \longrightarrow$ L-leucine + NAD^+ (1a)

NAD^+ + L-malate $\longrightarrow$ pyruvate + CO_2 + NADH (1b)

The course of the reaction was monitored by HPLC. The
result can be seen from the following table 1 in comparison
with an equivalent conversion with FDH instead of MDH.

Table 1: Comparison of the formation of L-leucine (HPLC) in
a coupled batch with coenzyme regeneration by MDH and FDH.
In each case 10 mM ketoisocaproate and for the regeneration
by malate dehydrogenase (MDH) 100 mM L-malate or by formate
5   dehydrogenase (FDH) 100 mM formate were employed.

Time [min]	L-Leucine with MDH [mM]	L-Leucine with FDH [mM]
0	0.7	0.1
10	8.8	9.9
30	9.6	11.1
60	10.8	9.8
120	9.9	9.9

The use of alcohol dehydrogenases in combination with
malate dehydrogenases was also investigated. The MDH from
E. coli is coupled with an NAD-dependent S-specific alcohol
10   dehydrogenase from *Rhodococcus erythropolis* (RE-ADH;
DE10218689). The usability of the MDH is tested here via
the reduction of a ketone (p-Cl-acetophenone = pCAp) in
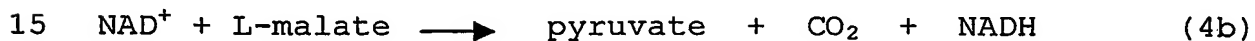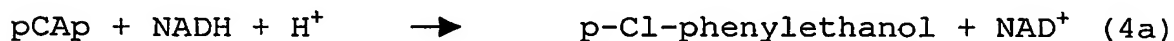accordance with equation (4).

$$pCAp + NADH + H^+ \longrightarrow p\text{-}Cl\text{-}phenylethanol + NAD^+ \quad (4a)$$

15   $$NAD^+ + L\text{-}malate \longrightarrow pyruvate + CO_2 + NADH \quad (4b)$$

Table 2: Decrease in the ketone p-Cl-acetophenone (employed
10 mM) and increase in the enzymatically formed alcohol p-
Cl-phenylethanol (100%, corresp. to 10 mM) as a function of
time.

Time [min]	Ketone [%]	Alcohol [%]
0	100	0
10	28	72
20	12	88
30	4	96
60	0	100

5

Enantiomerically enriched or enantiomer-enriched describes
the fact that one optical antipode is present in a mixture
with its other to >50%.

If one stereo-centre is present the structures shown relate
10   to the two possible enantiomers, and if more than one
stereo-centre is present in the molecule they relate to all
the possible diastereomers and, in respect of a
diastereomer, to the two possible enantiomers of the
compound in question which fall under this.

15   The organism *Candida boidinii* is deposited under number
ATCC 32195 at the American Type Culture Collection and is
accessible to the public.

The documents of the prior art mentioned in this
specification are regarded as also included in the
20   disclosure.

Descriptions of the drawings:

Fig. 2 shows a membrane reactor with dead-end filtration.
The substrate 1 is transferred via a pump 2 into the
reactor space 3, which contains a membrane 5. In the
5   reactor space, which is operated with a stirrer, are, in
addition to the solvent, the catalyst 4, the product 6 and
unreacted substrate 1. Low molecular weight 6 is chiefly
filtered off via the membrane 5.

Fig. 3 shows a membrane reactor with cross-flow filtration.
10   The substrate 7 is transferred here via the pump 8 into the
stirred reactor space, in which is also solvent, catalyst 9
and product 14. A solvent flow which leads via a heat
exchanger 12, which may be present, into the cross-flow
filtration cell 15 is established via the pump 16. The low
15   molecular weight product 14 is separated off here via the
membrane 13. High molecular weight catalyst 9 is then
passed back with the solvent flow, if appropriate via a
heat exchanger 12 again, if appropriate via the valve 11,
into the reactor 10.

20

Example 1:

For production of the MDH from *E. coli* K12 used here, see
the dissertation by S. Naamnieh, University of Düsseldorf,
in preparation, p. 70 et seq.

5   Purification and biochemical properties of the MDH from E.
coli:

a) Purification

The purification of the recMDH from *E. coli* crude extracts
(expression strain: *E. coli* derivative JM105) was carried
10 out in accordance with purification protocol (Stols L., and
Donnelly M. I. (1997). Production of succinic acid through
overexpression of NAD(+)-dependent malic enzyme in an
Escherichia coli mutant. *Appl Environ Microbiol* **63**: 2695-
701.). The rec-bacteria cells were first broken down by
15 disintegration with glass beads (breakdown buffer Tris/HCl
100 mM pH 7.5). Thereafter, a purification step by a Q-
Sepharose was carried out. After the purification by means
of Q-Sepharose, it was possible to determine a specific
activity of the MDH of about 7.3 U/mg. By purification of
20 this enzyme by means of further chromatography steps,
hydroxyapatite and Phenylsepharose, it was possible to
purify the MDH to homogeneity with a specific activity of
133 U/mg.

Tab. 3: Summary of the purification of the rec-MDH from *E. coli.*

Purification step	Activity (U)	Protein (mg)	Spec. act. (U/mg)	Enrichment (-fold)	Yield (%)
Crude extract	210	202	1.03		100
Q-Sepharose	184	25.2	7.3	7.1	88
Hydroxy-apatite	68	1.6	42.5	41	32
Phenyl-sepharose	24	0.18	133	129	11

b) Biochemical characterization

5   - Km values

For L-malate a Km value of 0.29 mM was measured, and for the coenzyme NAD^+ a Km value of 0.14 mM.
Both Km values lie in a low range of < 1 mM, and they show that the two substrates are recognized by the enzyme with a
10   good affinity. The two values suggest that the MDH can be used for the regeneration of NADH.

- Optimum pH (fig. 1)

MDH shows a maximum activity at relatively high pH values of 11 and higher. Nevertheless, the drop in activity at
15   lower pH values is relatively small, thus 72% activity is still present at pH 8.0, and 67% activity still at pH 7.0.

- Optimum temperature

The optimum temperature of the MDH is approx. 55°C (fig. 4).

Example 2:

5   a) Coupling of leucine dehydrogenase with malate dehydrogenase

The MDH from E. coli is coupled with an NAD-dependent leucine dehydrogenase (LeuDH from *Bacillus cereus*; Sigma). LeuDH catalyses the reductive amination of aliphatic keto
10   acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH.

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in
15   parentheses):

526 µl Hepes buffer (200 mM Hepes, pH 8.5 with 10 mM MgCl$_2$); 143 µl ammonium sulfate solution (500 mM in the test); 100 µl ketoisocaproate (100 mM); 20 µl NAD$^+$ (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes
20   buffer, pH 8.5); 1 µl LeuDH (0.5 U in the test); 10 µl MDH (1.5 U in the test; partly purified).

The test batch is incubated at 30°C, and after 0, 10, 30, 60 and 120 min samples are taken (50 µl; Eppendorf reaction vessels) and heated for 3 min at 95°C to stop the reaction.
25   Denatured protein is separated off by centrifugation for 10 min at 13,000 rpm (Eppendorf bench centrifuge) and the supernatant is analysed by means of HPLC, after derivatization with ortho-phthalaldehyde (OPA).

Derivatization with OPA (= ortho-phthaldialdehyde):

30   140 µl Na borate buffer (100 mM; pH 10.4); 40 µl sample or standard; 20 µl OPA/IBLC reagent (= ortho-phthaldialdehyde

/ N-isobutyryl-L-cysteine). 20 µl of this reaction solution are injected for the HPLC analysis.

HPLC analysis:

For the results see table 1:

5

b) Comparison experiment: Coupling of leucine dehydrogenase with formate dehydrogenase

In a comparison batch carried out in parallel, the same components as described above were used, but instead of the
10   malate dehydrogenase 0.5 U formate dehydrogenase (FDH from *Candida boidinii;* Sigma) was employed and instead of 100 mM L-malate 100 mM formate was used as the regeneration substrate.

For the results see table 1.

15

Example 3: Coupling of the MDH with alcohol dehydrogenase:

The MDH from *E. coli* (expression strain: *E. coli* derivative JM 105) is coupled with an NAD-dependent S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH). The
20   usability of the MDH is tested here via the reduction of a ketone (p-Cl-acetophenone).

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in parentheses):

25   678.7 µl Hepes buffer (100 mM Hepes, pH 8.5 with 10 mM $MgCl_2$); 1.3 µl p-Cl-acetophenone (10 mM in the test); 20 µl NAD^+ (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes buffer, pH 8.5); 15 µl RE-ADH (1 U in the test); 85 µl MDH (1 U in the test; partly purified).

The test batch is incubated at 30°C, and after 0, 10, 20,
30 and 60 min samples (50 μl) are taken, 100 μl ethyl
acetate are added and the upper phase is analysed by means
of gas chromatography for the formation of the alcohol p-
5 Cl-phenylethanol.

For the results see table 2.

Example 4: Construction of an expression vector with
heterologous expression

10 From the sequence of the amplified fragment, primers with
integrated restriction cleavage sites and a codon for the
ribosomal binding site were constructed. After
amplification of the malate dehydrogenase from the
recombinant pUC18, the PCR fragment was cloned into the
15 recombinant recPhe-pKK-223-3 expression vector after the
PheDH sequence at the PstI and HindIII restriction cleavage
sites (fig. 8 - Construction of the plasmid for a
heterologous expression for L-Phe synthesis by means of
whole cell conversion).

20 PCR:

5' forward: N'-malic-pst
5' CTGCAGAGCCCAGGGATGGATATTCAAAAA 3'
concentration 100 pmol/μl

5' reverse: C'-malic-Hin
25 5' AAGCTTTTAGATGGAGGTACGGCGGTAGTC 3'
concentration 100 pmol/μl

Table 4: PCR protocol for amplification of the malate dehydrogenase from the recombinant pUC18 plasmid. The concentrations of the template DNA were varied.

Template DANN recpUC18	N' -malic- pst prim 1	C' -malic- Hin prim 2	dNTP	Buffer	Taq polymerase	H₂O
50 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl
25 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl
10 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl

5

One cycle consists of:

Denaturing step :   94°C
Annealing step: 59°C
Amplification step: 72°C

10   Cloning:

The new construct of the recombinant plasmid (fig. 5) was transformed into competent E. coli cells JM 105 or HB 101.

The standard transformation was carried out in accordance with the protocol of Hanahan (Hanahan D. (1983). Studies on
15   transformation of Escherichia coli with plasmids. J Mol Biol 166: 557-80.). For this, 100-200 µl of competent E. coli cells were thawed on ice and 40 ng DNA from the ligation batch were added. The plasmid cell suspension was cooled for 30 min on ice and then heated at 42ºC for 90 sec
20   and immediately cooled again on ice. After addition of 300 µl LB medium the cells were incubated for about 45 min at 37ºC for regeneration. 200 µl of this culture were then

plated out on to an LB plate containing antibiotic and
incubated overnight at 37ºC.

With the cloning of the malate dehydrogenase 3' to the
PheDH on the PstI and HindIII cleavage site with its own
5   ribosomal binding site, expression of the two enzymes could
take place simultaneously.
Note: Further detailed information on the experimental
preparation of these cells will be described in: S.
Naamnieh, *Dissertation*, University of Düsseldorf, in
10 preparation.

Example 5: Coexpression of PheDH and malate dehydrogenase

Of the positive clones, some were selected for expression
An individual colony of the particular clones was
15 transinoculated into 5 ml LB$_{amp}$ medium, and after OD$_{580}$ = 0.6
was reached was induced with 1 mM IPTG. The induction was
carried out overnight and the harvested cells were broken
down with ultrasound.

The recombinant strain HB101 shows a malate dehydrogenase
20 activity of 100 U/ml and likewise a PheDH activity of
130 U/ml. The activity of the two enzymes in the
recombinant strain JM 105 is clearly higher and is at
~ 600 U/ml for malate dehydrogenase and ~ 1,200 U/ml for
the PheDH.

25 The two recombinant strains were cultured in a 10 l
fermenter and the activity of the two enzymes was
determined.

Table 5: Determination of the activity of the expressed
enzymes in a 10 l fermenter with LB medium as batch
fermentation.

	Activity of PheDH		Activity of malate dehydrogenase	
	(U/ml)	(U/mg)	(U/ml)	(U/mg)
E. coli HB101	400	33	220	22
E. coli JM105	1300	118	640	71

5   It can be seen from the expression data that the *E. coli*
    strain JM 105 shows a significantly better activity for
    both enzymes, and all further experiments were therefore
    carried out with this strain.

10 Example 6: Optimization of the activity

    To be able to utilize the maximum activity of the crude
    extracts obtained, several parameters were investigated and
    varied.

    The two heterologously expressed enzymes show the best
15 stability properties under different conditions. It was of
    interest to determine the optimum properties for both
    enzymes in the same system. The following experiments were
    carried out with a view to this fact.

    a) Optimization of the breakdown buffer

20 1 g of cells JM_{105} was broken down in 0.1 M Tris or 0.1 M
    Kpi buffer with/without BSA (1.5 g/l) 30%. The breakdown
    was carried out with ultrasound over 70 cont. cycles. In

addition to the buffers, 1.5 g/l BSA were added for stabilization of the enzymes.

Table 6: Comparison of the activity as a function of the breakdown buffer

	0.1 M Tris		0.1 M Kpi	
	− BSA	+ BSA	− BSA	+ BSA
PheDH	520 U/ml	610 U/ml	730 U/ml	1100 U/ml
Malate dehydrogenase	430 U/ml	720 U/ml	320 U/ml	610 U/ml

5

The addition of BSA led to an increase in the activity in both cases. The breakdown buffer also influenced the activities. It was to be seen here that the suitable breakdown buffer was different for the individual enzymes.

10   The Kpi buffer was more suitable for the PheDH than for the malate dehydrogenase, but since the decrease in activity of the malate dehydrogenase in the Kpi buffer was relatively low, the recombinant cells continued to be broken down in this buffer after the heterologous expression.

15

b) Duration of breakdown to investigate the stability

The duration of the breakdown was also investigated and the critical point for stability of the enzymes during this operation was determined. The optimum duration of breakdown
20   can be obtained from these data (fig. 9 – Stability determination of the PheDH and the malate dehydrogenase after various breakdown times by means of ultrasound. The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.

The following suspension was used for this experiment:

1 g recombinant cells (JM105)
3 ml Kpi buffer 0.1 M

During longer treatment of the cells with ultrasound the
activity of the PheDH decreases drastically, whereas the
activity of the malate dehydrogenase is retained. The ideal
breakdown conditions for a 25% breakdown of 1 g of
recombinant *E. coli* JM105 are therefore 4 x 30 s ultrasound
treatment with 3 x 30 s intermediate cooling in an ice-
bath.

During longer treatments with ultrasound the sample is
heated, which can lead to denaturing of the enzymes. The
amount of protein was determined in this experiment and can
be seen from the following.

Table 7: Protein determination of the two enzymes
expressed, PheDH and malate dehydrogenase, after variation
of the breakdown time. The cells were cooled intermediately
for 30 seconds after a 60 and 30 second treatment.

	60 sec x 2 spec. activity: U/mg	30 sec x 4 spec. activity: U/mg	30 sec x 8 spec. activity: U/mg
PheDH	30	105	16
Malate dehydrogenase	20	90	110

With a purification of the malate dehydrogenase to
homogeneity, it was possible to achieve a specific activity
of 466 U/mg. The purification steps are summarized in table
8.

Table 8: Purification of the recombinant malate
dehydrogenase

	Volume (ml)	Activity (U)	Protein (mg)	Spec. activity (U/mg)	Yield (%)	Factor
Ultracen-trifugation	0.6	820	13	63	100	1
Hydroxy-apatite	9	470	5	94	57	1.49
Q-Sepharose	7	496	2.1	236	60	3.74
Phenyl-sepharose	1.9	280	0.6	466	34	7.39

c) Km value determination

5   The K_M values were determined for the substrate and the
     coenzyme of the malate dehydrogenase. The K_M values were
     determined on homogeneous or partly purified rec-malate
     dehydrogenase samples.

     L-Malate: 0.29 mM
10   NAD$^+$ 0.14 mM

     Example 7: Coupled L-phenylalanine synthesis with
     regeneration of the coenzyme NADH

     Important factors for a coupled reaction are the optimum pH
15   and the heat stability of the two enzymes. In addition to
     the stability of the enzymes, further factors play a role,
     such as e.g. the influence of the various substrates on the
     enzymes.

In respect of the optimum pH, a kinetics study was
conducted and the pH-dependency of the synthesis was
determined. The increase in the activity at an increasing
pH can be seen from fig. 10, a pH of 8.0, in which although
5   the two enzymes do not show the highest activity, the
coenzyme remains, stable, being chosen for the synthesis for
coenzyme stability reasons (fig. 10 - Optimum pH of malate
dehydrogenase and PheDH. The activity of the two enzymes
increases as the pH increases. The measurements were
10   carried out with partly purified enzyme. For the
phenylalanine dehydrogenase, the reductive amination was
measured).

A second factor for the coupled enzyme reaction is the
suitable temperature at which the two enzymes remain stable
15   for a relatively long period of time. A further experiment
was therefore carried out to determine the optimum
temperature (fig. 11 - Optimum temperature. The
measurements were carried out at pH 8.5 and in 0.1 M HEPES
buffer).

20   As can be seen from fig. 11, the optimum temperature of
both enzymes is 50°C. At 30°C the activity measured is only
60%.

The malate dehydrogenase is stable at 45°C for a relatively
long period of time. However, since the PheDH becomes
25   unstable at this temperature value, the syntheses were
carried out at 30°C, so that it was possible to ensure the
stability of both enzymes and of the coenzyme over a
relatively long period of time.

Enzymes achieve their optimum activity in the particular
30   suitable buffer. The two enzymes were tested with two
different buffers in each case in a reaction batch (table
9).

Table 9: Comparison of the PheDH and malate dehydrogenase
.activity in various reaction buffers. The activities are to
be seen in per cent of the optimum.

	0.1 M HEPES buffer (pH 8.0)	0.1 M Tris buffer (pH 8.0)
PheDH	84 %	100 %
MDH	100 %	42 %

5   Since the activity of the PheDH in HEPES buffer does not
decrease considerably, the coupled enzyme reaction was
carried out in this buffer.

With the determination of the buffer, pH and temperature
values, it was possible to select suitable conditions and
10   media for the synthesis of phenylalanine by a coupled
enzyme reaction with regeneration of the cofactor (NADH).

30 mM phenyl pyruvate, 100 mM ammonium sulfate, 100 mM
HEPES buffer, 70 mM L-malate, 2 mM NAD$^+$, 2 mM Mg $^{2+}$, 25 U
PheDH (partly purified) and 30 U malate dehydrogenase were
15   employed. The samples are analysed by means of HPLC.
The synthesis was monitored for several hours. After 4 h
approx. 50% of the substrate employed, phenyl pyruvate, was
converted into L-phenylalanine (fig. 12 – Formation
kinetics for L-Phe. The formation of L-Phe was carried out
20   *in situ*).

Example 8: Whole cell conversion

The following medium was used as the standard batch for the
whole cell conversion:

0.1 M	HEPES buffer (pH 8.0)
40 mM	phenyl pyruvate
0.1 M	L-malate
0.1 M	ammonium sulfate
2 mM	$MgCl_2$

The conversion was carried out at 30°C and with 1 g of recombinant *E. coli* cells. The phenylalanine formed was detected by means of HPLC (fig. 6).

The formation of L-Phe by recombinant *E. coli* cells was monitored for 20 h and the yield was determined (fig. 7).

No metabolization of the product formed, L-Phe, was to be detected after incubation for 20 h.